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Original Article

Effect of Harvesting Time on Content and Chemical Composition of Essential Oil from *Stachys lavandulifolia* Vahl (Lamiaceae)

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Abstract

Stachys L. one of the biggest genus of the Lamiaceae family comprises about 200-300 species. In this research, aerial parts essential oil composition and content of *Stachys lavandulifolia* Vahl (Lamiaceae) at different stages (vegetative, full flowering and initial fruiting stages) is reported. The oils obtained by water distillation method (Clevenger apparatus) and analyzed by GC and GC/MS. The results showed that the essential oils of *S. lavandulifolia* were affected by plant growth stages. The chemical composition varied in three harvesting times. It was found that the maximum obtained essential oil was in the flowering stage. Totally, 31 constituents with the range of 0.10 – 34.11% in the vegetative, 27 constituents with the range of 0.06 – 36.35% in the flowering stage and 27 compounds with the range of 0.06 – 37.2% in the initial fruiting stages were identified. The highest compounds were related to the vegetative stage (34 compounds) that representing 63.74% of oil. In this study, the highest amount of essential oil constituents in the vegetative stage belonged to germacren D (34.11%), n-decane (3.84%) and caryophyllene oxide (2.62%), in the flowering stage, germacren D (36.87%), borneol (4.3%), cis-thujone (4.24%), bicyclogermacrene (4.16%) & n-decane (3.88%) and in the stage of initial fruiting, germacren D (37.2%), borneol (4.76%), β-caryophyllene (4.20%), cis-thujone (4.16%) & bicyclogermacrene (3.99%).

Keywords: *Stachys lavandulifolia,* Germacren D, Essential oil

Introduction

Stachys L. is one of the Lamiaceae family that comprises about 200-300 species, one of the largest genus of this family is considered [1,2]. Iran has 34 species of this genus, among which, 13 are endemic [3]. *Stachys* are distributed mainly in warm regions of the Mediterranean and Southwest Asia, Southern Africa, South and North America [4,5]. These plants are grown under various ecological conditions in habitats like rocky places, mountain steppes and stream banks or sometimes in forests [6]. *S. lavandulifulia* is known as Chay-e-kohi (Mauntain tea) in Persian and Betony in English [7]. *S. lavandulifulia* in Iranian folk medicine is used for treat diarrhea, anxiolytic and sedative [8]. This plant is used as the herbal tea in gastrointestinal disorders, inflammatory diseases, cough, antispasmodic, diuretic, ulcers and fevers [7]. The decoction of the leaves and flowers is being used by the tribal people of Chaharmahal va Bakhtiari for treatment of skin infection, menorrhagia and anti-bacterial [9]. Phytochemical investigations established the presence of various mono- and sesquiterpenes in its essential oils [10, 11] and phenylethanoid glycosides, verbascoside and lavandulifoliaside in the plant extract [12]. This genus contains different natural product classes,

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including monoterpenes, sesquiterpenes, diterpenes [13-15], saponins [16], flavonoids, bioflavonoids, glycosides and phenolic acid [17]. The essential oil of aerial part of *S. lavandulifolia* was also analyzed by GC/MS method and germacrene-D (13.2%), β-phellandrene (12.7%), β-pinene (10.2%), myrcene (9.4%), α-pinene (8.4%), as well as Z-β-ocimene (5.8%) reported as main components of the essential oil [11]. The main components in the essential oil of *S. byzantine* were identified piperitenon (9.9%), 6,10,14-trimethyl pentadecan-2-one (6.4%) and *n*-tricosane (6.4%) [18]. Forty-seven components (95.1%) were identified in *S. inflate* essential oil, which major components were hexadecanoic acid (9.1%), germacrene D (8.9%), α-pinene (5.8%) and bicyclogermacrene (5.1%) [19]. In the essential oil of *S. laxa*, germacrene D (17.1%), 4-hydroxy-4-methyl-2-pentanone (12.3%), 7-*epi*-α-selinene (8.3%), bicyclogermacrene (6.7%), β-caryophyllene (6.2%) and α-pinene (5.9%) were found as the main constituents [18]. Some studies have reported that the amount and compositions of essential oil in the plants are affected by different stages of growth [20, 21]. The highest yield of essential oil of *Thymus daenensis* was in full flowering stage (2.28%) [21]. The most oil was contained in Hyssop (*Hyssopus officinalis* L.) from plant in full blooming (1.7%) and the lowest level of essential oil was found in vegetative phase (0.6%) [20]. Also, this has been demonstrated for *Dracocephalum moldavica*, *Thymus capitatus*, *Artemisia judaica* and *Thymus vulgaris* [22-25]. This research deals with the results of the chemical analysis of the oils obtained from different phenological stages of *S. lavandulifolia* including vegetative, flowering and fruiting stages.

A literature survey showed that the harvesting time effected on the chemical composition of different plants. This can help to select the best harvesting time to obtain the highest percentage of essential oil. Due to medicinal properties of *S. lavandulifolia*, the aim of the present research was to determine the best time to harvest plant from Shohada Valley in West Azerbaijan that obtains maximum performance.

Material and Methods

The aerial parts of *S. lavandulifolia* were collected in May, June and July 2016 at different phenological stages: vegetative (beginning of May), flowering (beginning of June) and fruiting (beginning of July) stages from its natural habitat, Shohada-Valley of the Urmia city in Western Azerbaijan Province, Iran (Table 1). The collected plants were identified in the Department of Medicinal Plant, Urmia University, Iran based on the botanical reference of Ghahreman, 1979-1992 [26].

Essential Oil Extraction

The aerial parts of plant in different stages were dried in herbarium, Urmia University, Iran. According to the recommended method by the European Pharmacopoeia, the powdered samples (50 g) were extracted by water hydro-distillation for 3 hours [27]. The extracted EOs samples were dried over anhydrous Na_2SO_4. Finally, the extracted samples were stored in sealed vials at low temperature (4 ˚C) until analysis by gas chromatography (GC) and gas chromatography/mass spectrometric (GC/MS).

GC and GC/MS Analysis

The analysis of EOs was carried out using a gas chromatography (GC), which was performed using a Shimadzu model A9 equipped, with a DB-5 (dimethylsiloxane, 5 % phenyl) fused silica capillary column (30 m×0.25 mm i.d., film thickness 0.25 µm). EOs (100 µl) was injected while Helium with purity of 99.999 % was used as a carrier gas at a pressure of 1.5 $kgcm^2$ and flow rate of 31.5 cms^{-1}. Thermal planning of column was started at 60 ˚C and then programmed to rise to 210 ˚C at a rate of 3 ˚C min^{-1}. After raising the temperature to 210-240 ˚C at a rate of 20 ˚C min^{-1}, stay at this temperature for 8.5 min. The flame ionization detector (FID) and injector temperature was 280 and 300 ˚C, respectively. EOs was also subjected to gas chromatography/mass-spectrometric (GC/MS) analyses using a Varian 3400 GC/MS system. The GC/MS was equipped with a DB-5 column (30 m × 0.25 mm i.d., film thickness 0.25 µm).

Table 1 The geographical features of the studied area

Latitude	Longitude	Altitude (m)	Temperature (˚C)	Climate	Annual rainfall (ml)

37˚ 18' 45" -37˚ 17' 05" N	45˚ 6' 35" -45˚ 07' 30" E	1400–2010	11.89	cold semi dried	281.72

Oven temperature was the same as the previous one. The final temperature of injection chamber was adjusted 10 ˚C higher than column temperature. Carrier gas was helium with a flow rate of 31.5 cm s^{-1}, scan time was 1 s, the ionization energy was 70 eV, and mass range 40-340 AMU (atomic mass unit).

Identification of EOs Components

The constituents of the essential oils were identified by calculation of their retention index. The linear retention index for all compounds was determined by injection of the sample with a solution containing the homologous series of n-alkanes. Retention index were determined using retention times of n-alkanes that were injected before the essential oil under the same chromatographic conditions. The compounds were identified by comparison of retention indices with those reported in the literature and by comparison, of their mass spectra with the Wiley GC/MS Library and Adams Library data published mass spectra data [28].

Results and Discussion

In this research, the chemical composition of the aerial parts of *Stachys lavandulifolia* Vahl was analyzed by GC and GC-MS (Table 2). The vegetative stage was characterized by the lowest amount of essential oil yield about 0.3%, during the flowering stage, the oil content increased rapidly to be 3.2%. In the fruiting stage, the oil content decreased (2.5%). Therefore, it was found that the maximum essential oil production occurred in the flowering stage. Totally, 31 constituents with the range of 0.10–34.11% in the vegetative, 27 constituents with the range of 0.06–36.35% in the flowering stage and 27 compounds with the range of 0.06 – 37.2% in the initial fruiting stages were identified. The highest compounds were related to the vegetative stage, so 31 compounds were identified that representing 63.74% of oil. In this study, the highest amount of essential oil constituents in the vegetative stage belonged to germacren D (34.11%), n-decane (3.84%) & caryophyllene oxide (2.62%), in the flowering stage, germacren D (36.87%), borneol (4.3%), cis-thujone (4.24%), bicyclogermacrene (4.16%) & n-decane (3.88%) and in the stage of fruiting, germacren D (37.2%), borneol (4.76%), E-caryophyllene (4.20%), cis-thujone (4.16%) & bicyclogermacrene (3.99%) (Table 2). The results showed that the essential oils of *S. lavandulifolia* were affected by plant growth stages and there were differences among the oil of *S. lavandulifolia* in quantity and quality in phenological different stages. Based on other studies this demonstrated in other plants [3,9,16,24,29,30].

Germacrene D (a sesquiterpene, $C_{15}H_{24}$), had the highest content in three harvesting times and was reported to have cytotoxicity effect [31].

The percentage and number of different chemical classes of *S. lavandulifolia* essential oil are given in table 3.

The major monoterpenoids compounds in vegetative stage were 1- octane (1.38%), cineole (0.47%), cis-thujone (0.72%) and camphor (0.34%), but the major sesquiterpenoids components were germecrene D (34.11%) and E-caryophyllene (2.48%). The major monoterpenoids compounds in flowering stage were borneol (4.27%), cis-thujone (4.24%) and 1,8-cineole (3.70%) & the major sesquiterpenoids components in this stage were germacrene D (36.87%), bicyclogermacrene (4.16%) and E-caryophyllene (3.86%). The major monoterpenoids components in fruiting stage were borneol (4.76%) and cis-thujone (4.16%). The major sesquiterpenoids components were germacrene D (37.21%), borneol (4.76%), E-caryophyllene (4.20%), cis-thujone (4.16%) and 1,8-cineole (3.49%). These results could be explained that the date of harvest has a large effect on the essential oil components studied. Meshkatalsadat *et al* (2007) indicated that the essential oil of *S. lavandulifolia* consisted mainly of monoterpene hydrocarbons (pre-flowering: 72.89%, flowering: 80.43%, and post-flowering: 69.42%), oxygenated monoterpenes (pre-flowering: 0.36%, flowering: 0.68% and post-flowering: 0.28%), sesquiterpene hydrocarbons (pre-flowering: 21.78%, flowering: 16.09% and post-flowering: 26.64%), oxygenated sesquiterpenes (pre-flowering: 3.84%, flowering: 1.42% and post-flowering: 2.48%), oxygenated diterpenes (pre-flowering: 0.13%, flowering: 0.52% and post-flowering: 0.09%) [8]. These results confirm with the present results that monoterpenes were the major portion of all population. The study of Shahnama *et al*. (2015) on *S. lavandulifolia* at flowering period showed compounds myrcene (41.55%), α-pinene (33.3%), β-terpinene (12.16%),

β-pinene (5.06%), sabinene (1.34%) and α-phellandrene (1.27%) [32].

Table 2 Essential oils (EOs) and chemical composition of *Stachys lavandulifolia* Vahl during three harvesting times.

Components	RI	Vegetative stage	Flowering stage	Fruiting stage
1 – octane	788	1.386	0.556	-
α-Pinene	932	0.766	1.226	1.466
Camphene	946	-	0.316	0.426
Benzaldehyde	952	0.126	-	-
sabinene	969	0.316	0.496	0.506
β-Pinene	974	0.1	0.066	0.626
n-Decane	1000	3.846	3.886	4.033
1,8-cineole	1026	0.476	3.706	3.496
cis- Thujone	1101	0.726	4.243	4.166
Ttrans – Thujone	1112	0.136	0.116	0.066
Camphor	1141	-	1.246	1.256
Borneol	1165	0.166	4.276	4.769
n-decanal	1201	0.866	-	-
Bornyl acetate	1284	1.266	0.896	-
α-Copaene	1374	0.496	-	-
β- cubebene	1387	0.736	-	0.896
β-Bourbonene	1387	0.316	0.446	0.986
β-Elemene	1389	0.686	0.466	2.246
n-Tetradecane	1400	0.496	-	-
(E)- Caryophyllene	1417	2.486	3.866	4.206
(Z)- β- Farnesene	1440	-	0.886	1.246
(E)- β- Farnesene	1454	0.476	-	-
9-epi- (E)- Caryophyllene	1464	-	0.766	-
Germacrene D	1484	34.116	36.87	37.21
(2E, 6E)-Farnesol	1742	1.386	-	0.226
Bicyclogermacrene	1500	1.056	4.166	3.996
(E) –γ- Bisabolene	1529	0.216	0.316	0.406
α-Calacorene	1544	0.346	1.066	1.480
Spathulenol	1577	-	2.466	2.866
Caryophyllene oxide	1582	2.626	3.356	2.976
α-Muurolol	1644	0.476	0.416	0.406
α-Cadinol	1652	1.306	2.766	2.496
n- Tetradecanol	1671	0.166	0.656	0.966
(5E, 9Z)-Farnesyl acetone	1886	1.386	-	0.226
n-Nonadecane	1900	0.396	-	-
Heptacosane	2700	0.766	0.5	1.466

RI: Retention Index on DB-5 Column

Table 3 The percentage and number of different chemical classes of *Stachys lavandulifolia* Vahl essential oil.

Chemical classes	Vegetative		Flowering		After flowering	
	Number	Percentage	Number	Percentage	Number	percentage
Monoterpene	7	2.69	9	15.69	9	16.77
Sesquiterpene	14	46.72	13	57.85	14	61.68
Non-terpene	10	10.7	4	6.72	4	6.69
Total percentage	60.11		80.26		85.14	

47 components (95.1%) were identified in *S. inflate* oil, with major compounds n-hexadecanoic acid (9.1%), germacrene D (8.9%), α-pinene (5.8%) and bicyclogermacrene (5.1%) [18]. 63 components were identified in the oil of *S. lavandulifolia*, which comprised of 97.5% of its total composition. Major compounds were 4-hydroxy-4-methyl-2-pentanone (9.3%), α-pinene (7.9%) and hexadecanoic acid (5.2%) [18]. 34 compounds (97.3%) were identified in *S. laxa* essential oil, which major

components were germacrene D (17.1%), 4-hydroxy-4-methyl-2-pentanone (12.3%), 7-*epi*-α-selinene (8.3%), bicyclogermacrene (6.7%), β-caryophyllene (6.2%) and α-pinene (5.9%) [18]. The essential oil of *S. byzantine* comprised of 12 monoterpenoids (27.4%), 17 sesquiterpenoids (32.9%), 4 diterpeoids (7.0%) and 27 non-terpenoids (31.4%) [18]. The essential oil of *S. inflata* comprised of 18 monoterpenoids (31.0%), 15 sesquiterpenoids (36.5%), one diterpenoid (0.8%) and 13 non-terpenoids (26.8%) [18]. The oil of *S. lavandulifolia* comprised of 12 monoterpenoids (19.6%), 26 sesquiterpenoids (42.9%), three diterpenoids (2.1%) and 22 non-terpenoids (32.9%) [18]. The essential oil of *S. laxa* comprised of 9 monoterpenoids (17.3%), 18 sesquiterpenoids (60.0%), one diterpenoid (0.6%) and 6 non-terpenoids (19.4%) [18]. Harmandar *et al*. (1997) reported the essential oil of *S. oblique* L. consisted of 5 monoterpenes hydrocarbons (14.8%), 18 oxygenated monoterpenes (48.7%) and 6 sesqiterpenes (33.4%) [33]. In 2004, Sajjadi and Somae reported the major compounds of essential oil of *S. inflate* were germacrene D (16.9%), bicyclogermacrene (16.6%), α-pinene (11.3%), β-phellandrene (9.8%), β-pinene (5.6%) and spathulenol (3.2%) [34]. 79 compounds were identified representing 98.2% by Javidnia *et al.*, 2004, in the essential oil of *S. lavandulifolia*, which major components were germacrene D (13.2%), β-phellandrene (12.7%), β-pinene (10.2%) and α-pinene (8.4%) [11]. Hadipanah *et al.* (2015) reported 17 compounds for *S. lavandulifolia* with the major components α-pinene (49.24%), β-pinene (22.52%), β-phellandrene (11.71%), α-copaene (6.70%) and β-myrcene (2.20%) [35].

The main constituents of the essential oils of *S. lavandulifolia* collected throughout two provinces (Isfahan and Chaharmahal va Bakhtiary) were α-thujone (0.3%-32.3%), α-pinene (trace to 37.3%), myrcene (0.5%-15.9%), β-phellandrene (1.1%-37.9%), germacrene D (0.4%-11.3%), Δ-cadinene (trace to 11.6%) and 1, 4-methano-1 H-indene (trace to 10.1%) [36].

The result of chemical analysis of *Stachys serotina* (Host) Fritsch. essential oil by Jekovic *et al*. (2012) showed that sesquiterpene hydrocarbons were the most abundant class of isolated volatiles of β-caryophyllene, δ-cadinene, α-humulene and germacrene D [37]. In the study of Gorena *et al.* (2011) the essential oils from twenty-two different *Stachys* species have been identified thirty-nine compounds. Germacrene-D (2.9%- 45.3%), β-caryophyllene (2.3%-62.3%), caryophyllene oxide (trace to 7.8%), spathulenol (trace to 7.8%) and α-cadinene (1.4%-8.5%) have been identified as the main components of the essential oils [38].

Mirza and Baher (2003) reported that the oil of *Stachys lanata* Jacq. collected from the National Botanical Garden in Tehran, Iran, was rich in α-thujone (25.9%), α-humulene (24.9%), β-caryophyllene (12.6%) and viridiflorol (10.5%) [39]. In comparison with our results, compounds α-humulene and viridiflorol were not found in the essential oil of Shohada Valley. In addition constituents α-thujone and β-caryophyllene were of less abundance. In previous studies, the main components of *S. lavandulifolia* oil were reported to be germacrene-D (13.2%), β-phellandrene (12.7%), β-pinene (10.2%), myrcene (9.4%), α-pinene (8.4%) and Z-β-ocimene (5.8%) for Tehran population in Central Iran [11], myrcene (20.9%), α-pinene (16.3%), α-terpinene (20%) and bicyclogermacrene (8.7%) for Lorestan population in West Iran [40], α-pinene (7.9%), 4-hydroxy-4-methyl-2-pentanone (9.3%) and hexadecanoic acid (5.9%) for Mazandaran population in North Iran [18] and β-caryophyllene and 1,8-cineole for *S. lavandolifolia* in Turkey [41]. Comparison of the main components of the Shohada Valley *S. lavandulifolia* with those results showed that β-phellandrene, Z-β-ocimene, α-terpinene, 4-hydroxy-4-methyl-2-pentanone and hexadecanoic acid were not found in Shohada Valley oil sample at flowering stage.

Feizbaksh *et al*. found α-pinene (20.1%), β-pinene (12.1%), spathulenol (7.2%) and germacrene D (5.3%) as the major components of oil of *S. lavandulifolia* collected from Ab-ali (Tehran province, Iran) [10]; this oil was rich in monoterpenoids (51.8%). They identified 44 compounds with 51.8% monoterpenoids and 37.2% sesquiterpenoids. We confirm this result that Shohada Valley essential oil of *S. lavandulifolia* was rich of monoterpenoids, but α-pinene, β-pinene and spathulenol were less than Ab-Ali essential oil sample and also germacrene D was higher than it. In a study conducted by Morteza-semnani *et al* in 2006, about 60 compounds were identified in the essential oil of *S. byzantine* at flowering stage, representing about 98.7% of the total composition of the oil, which major components were piperitenone (9.9%), 6,10,14-trimethyl pentadecan-2-one (6.4%) and *n*-tricosane (6.4%) [18]. A high α-pinene (20.1%), β-pinene (12.1%) and

spathulenol (7.2%) in Farsan population has previously been proposed in the essential oil of *S. lavandulifolia* collected from Tehran, Iran [9]. In our study, these constituents are less than that report.

The major components of *S. lavandulifolia* oil from central Zagrus Mountains were β- phellandrene (37.93%), α-thujene (23.76%), benzaldehyde (6.28%), β-myrcene (4.41%), γ-elemene (3.98%) and bicyclogermacrene (2.64%) [4]. The main components of *Stachys lavandolifolia* were collected at the after flowering stage from the Darkesh Protected Area, Bojnourd (North Khorasan Province Iran) were bis (2- ethylhexyl) phthalate (58.39%), decane (25.46%), p-xylene (4.2%), dodecane (3.85%) and α-pinene (3.29%) [42].

The aerial parts of *S. lavandulifolia* were collected at full flowering stage from five natural geographical habitats during June 2011 (as Hurestaneh (Golpayegan), Siahdarreh (Fereydan1), Fereydunshahr, Afus (Fereydan 2) and Golestan Kuh (Khansar) located at Isfahan province (central parts of Iran). Results indicated that main components were germacrene-D (15.96%), thymol (14.64%), γ-cadinene (13.33%), α-pinene (7.80%) and *trans*-caryophyllene (6.91%). The materials gave yellow oil in a yield of 0.25% v/w [30].

The average yield of essential oil of *S. lavandulifolia* which collected after flowering stage was about 0.15% [42]. The yield of the oil obtained from *S. lavandulifolia* at full flowering stage was 0.25% [30]. Feizbakhsh *et al* (2003) gave 1.5% oil yield from *S. lavandulifolia* at flowering time [10]. Thus, the yield values of the oil in this study were, significantly, higher than those reported in previous literature data for *S. lavandulifolia*.

In a study that was done in Iran (Khoram Abad) at full flowering stage, 14 compounds were investigated and main compounds were α-terpinene (20%), α-pinene (16.3%), myrcene (20.9%). These findings were different in terms of number and kind of compounds with our results [40].

As a result, the variation in the essential oil composition could be attributed to both interactions between genetic (biotic) and environmental (abiotic) factors. According to this investigation, three phenological periods largely affected the amount of the major compounds.

Essential oil composition of *S. lavandulifolia* depends on many factors of genetic, environmental and their interaction effects, such as plant part, harvest-time, extraction-method, ecotype and geographic origin (climate, edaphic, elevation and topography) [41]. Genetic differences cannot be directly deduced from the varying amounts of a secondary plant product [34]. Plants growing in different environments grow ordinarily at different rates; they differ in size and developmental stage [43].

Conclusion

Results showed that in the vegetative, flowering and fruiting stages 34, 33 and 26 compounds were identified, respectively. Germacrene D (a sesquiterpene, $C_{15}H_{24}$), had the highest content in three harvesting times. The results showed that the essential oils of *S. lavandulifolia* were affected by plant growth stages and there were differences among the *S. lavandulifolia* essential oils in quantity and quality of the compounds in all studied population. The vegetative stage was characterized by the lowest amount of essential oil yield about 0.3%, during the flowering stage, the oil content increased rapidly to be 3.2%. In the fruiting stage, the oil content decreased (2.5%). Therefore, it was found that the maximum essential oil production occurred in the flowering stage.

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